Wound Healing in Membrane-Type-1 Matrix Metalloproteinase-Deficient Mice[1]

To the Editor:

Matrix metalloproteinases (MMP) are a family of at least 24 Zn^{2+}-dependent endopeptidases comprising secreted MMP and membrane-type MMP (MT-MMP). These enzymes have been implicated in wound repair by their ability to remodel extracellular matrix (ECM) components. Indiscriminate pharmacologic inhibition of MMP also blocks epithelial resurfacing emphasizing the physiological role of one or more MMP in wound healing (Lund *et al*, 1999; Ågren *et al*, 2001; Mirastschijski *et al*, 2002). Membrane-type-1 MMP (MT1-MMP) is an interesting candidate, due to its unique anchorage to the cell membrane, which facilitates localized pericellular proteolysis by the extracellular catalytic domain during keratinocyte movement (Seiki, 2002). In addition, MT1-MMP processes several cell surface-associated molecules and can regulate integrin cross-talk (Baciu *et al*, 2003).

The precise role of MT1-MMP in epithelial regeneration is unclear. Although MT1-MMP is expressed by normal human (Nagavarapu *et al*, 2002) and murine (Netzel-Arnett *et al*, 2002) epidermal keratinocytes, *in situ* hybridization studies failed to detect MT1-MMP gene expression in epithelium of human (Mirastschijski *et al*, 2002) or murine wounds (Madlener *et al*, 1998).

We have compared wound healing *in vivo* and epidermalization specifically *ex vivo* in neonatal MT1-MMP-deficient (MT1-$MMP^{-/-}$) mice with their normal littermates.

Generation of MT1-MMP mutant C57BL/6 mice are described elsewhere (Zhou *et al*, 2000). Following mating of heterozygotes, MT1-MMP-deficient (MT1-$MMP^{-/-}$), heterozygote (MT1-$MMP^{+/-}$), and wild-type (MT1-$MMP^{+/+}$) offspring were obtained in Mendelian ratio. The genotype of all animals used was verified with Southern analysis. The phenotype of heterozygous and wild-type mice is indistinguishable whereas the homozygous mutated genotype exhibits several developmental defects. Two to three litters, each comprising four to six pups, were kept per cage and breast fed. Lactating mice were given pellets and tap water *ad libitum*, and kept at 20°C and 50% relative humidity in sterile animal facilities. The local ethics committees of the Karolinska Institute (N 218-02) and Lund University (LU 508-99) approved the studies.

One full-thickness skin wound was made on the back by a sterile 4-mm punch biopsy in anaesthetized 3-d-old animals. Total content of hydroxyproline of excised skin biopsies did not differ significantly between normal (10.4 ± 0.8 μg, $n = 11$) and MT1-$MMP^{-/-}$ (10.9 ± 0.6 μg, $n = 8$) mice. This is in contrast to the observed skin fibrosis in MT1-MMP null mice indicative of excessive collagen accumulation (Holmbeck *et al*, 1999). The wounds were left uncovered for 7 d.

We first confirmed that wound healing in general was dependent on MMP in our neonatal mouse wound model by using the broad-spectrum MMP inhibitor GM 6001 that resulted in marked delayed wound closure (Fig 1*A*). In contrast, wound closure was unaltered in MT1-$MMP^{-/-}$ mice over 7 d (Fig 1*B*). The density of inflammatory cells or blood vessels in granulation tissue did not differ significantly between MT1-$MMP^{-/-}$ mice and their littermates over the 7-d period as evaluated semiquantitatively by light microscopy. Gelatin zymography showed increased levels of latent MMP-9 and MMP-2 post-wounding. MMP-9 was less abundant in MT1-$MMP^{-/-}$ relative to littermate wounds. The identity of mouse MMP-9 and MMP-2 was indicated by their positions corresponding to human MMP-9 and MMP-2. Although MT1-MMP is an important activator of MMP-2, the activation of pro-MMP-2 in MT1-$MMP^{-/-}$ non-wounded skin or wounds was only slightly reduced (Fig 1*C*). Addition of dithiothreitol to tissue extracts during electrophoresis abolished the MMP-2 lysis bands, indicating that other MMP at the positions of the MMP-2 forms were not detected in our zymographic assay. Another support for the identity of MMP-2 was that 4-aminophenylmercuric acetate treatment of tissue extracts resulted in a shift to lower-molecular-mass MMP-2 forms and accumulation of active MMP-2 (Fig 1*D*). The minor effect of MT1-MMP deficiency on pro-MMP-2 activation could be explained by the ability of the other five MT-MMP, including the glycosyl-phosphatidyl inositol-anchored MT-MMP, to activate latent MMP-2. Taken together, overlapping functions of the MT-MMP and other members of the MMP family may compensate for the absence of functional MT1-MMP *in vivo* and supports the concept of redundant activity among the MMP. In accordance with our results, other MMP knockout mouse strains display no or only minimal skin wound healing defects (Parks, 1999).

The fragility of the MT1-$MMP^{-/-}$ mice (Zhou *et al*, 2000) limited the open wound area accessible for epithelialization to about 5 mm^2 due to substantial wound contraction. To study centrifugal epidermalization over a larger surface area, fresh 2-mm skin explants from 3-d-old normal and MT1-$MMP^{-/-}$ mice were cultured *ex vivo* on an acellular human deepidermized dermal (DED) substrate. Laminin-1 immuno-

Abbreviations: DED, deepidermized dermis; ECM, extracellular matrix; MMP, matrix metalloproteinase; MT1-MMP, membrane-type-1 matrix metalloproteinase

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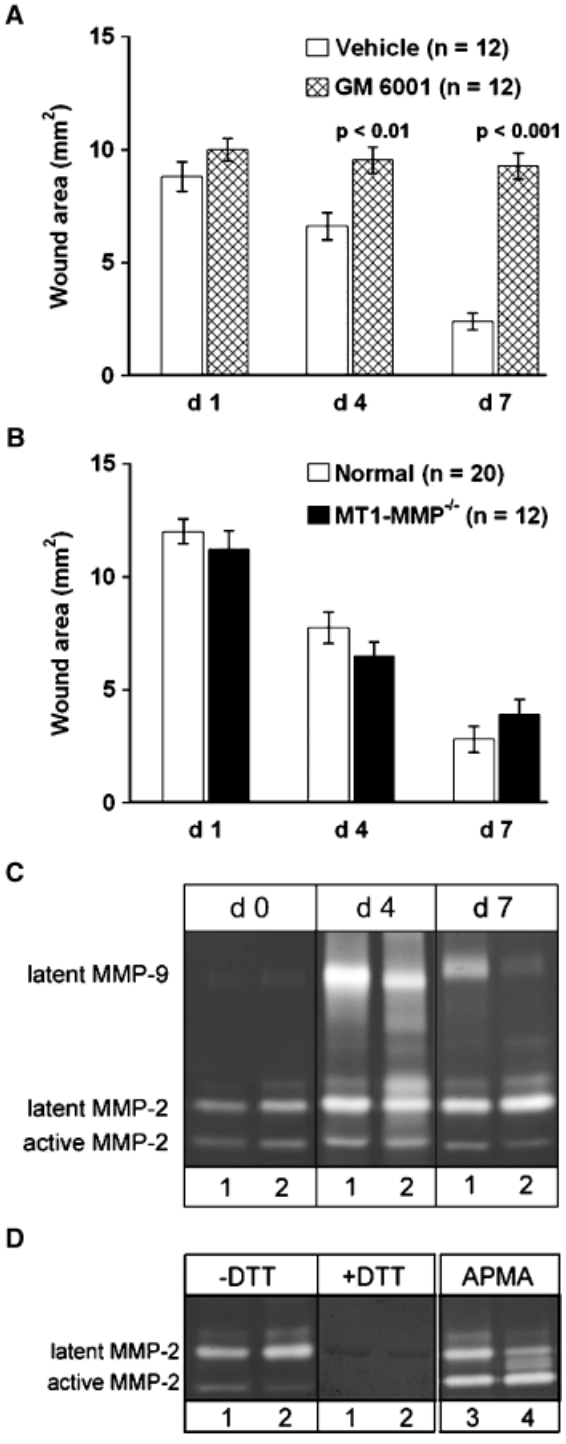

Figure 1
***In vivo* murine wound closure and zymography.** Wound closure in (*A*) normal 3-d-old mice injected subcutaneously and daily, starting 2 d prior to wounding, with the global hydroxamate matrix metalloproteinase (MMP) inhibitor GM 6001 (100 μg per g body weight) or vehicle alone (Lund *et al*, 1999) and in (*B*) littermate and membrane-type-1 matrix metalloproteinase-deficient (MT1-MMP$^{-/-}$) mice. Wound areas were determined by digital planimetry from tracings of outer wound margins and data analyzed by the *t* test. *Error bars:* SEM. (*C*) A representative gelatin zymogram of electrophoresed tissue extracts (15 μg total protein per lane), prepared in the presence of a proteinase inhibitor cocktail (Calbiochem, Darmstadt, Germany) for 18 h at 4°C (Mirastschijski *et al*, 2002), of normal skin (day 0) and wounds. (*D*) Zymograms of tissue extracts in the absence (−DTT) or presence (+DTT) of 50 mM dithiothreitol (DTT) in the sample buffer (Peeters-Joris *et al*, 1998), or after pre-treatment without or with 1 mM 4-aminophenylmercuric acetate (APMA) at 37°C for 2 h. (*C, D*) *1*, normal; *2*, MT1-MMP$^{-/-}$; (*D*) *3*, no APMA; *4*, APMA.

staining showed an intact basement membrane on the apical side of the DED substrate (Fig 2*A*) onto which the mouse skin explants were secured by fibrin and grown at the air–liquid interface for up to 9 d (Fig 2*B, C*). Negligible keratinocyte outgrowth was observed after ablation of 100 μm of the apical surface of DED in a cryomicrotome. The rate of epithelial outgrowth was assessed by exposing explants to 2.4 μM fluorescein diacetate with subsequent quantification of the fluorescent epithelium from digitized images (Lu and Rollman, 2004). After an initial phase of comparable outgrowth rates, epithelialization was retarded in the MT1-MMP$^{-/-}$ group when epithelial outgrowth reached 6–7 mm^2 (Fig 2*D–F*). In a separate but otherwise identical series, using skin explants from several different litters of mutant ($n = 30$) and normal mice ($n = 46$), impaired epithelial outgrowth ($p < 0.01$) from MT1-MMP$^{-/-}$ skin explants was reconfirmed as outgrowth areas approached 6–7 mm^2. Thus, the *in vivo* and *ex vivo* results may not be contradictory. Overall regeneration of neoepithelium is the net result of keratinocyte migration, proliferation, and apoptosis. We could not detect a significant involvement of MT1-MMP in cell proliferation (Fig 2*G–I*); however, the reduced formation of epithelium was linked to an increased proportion of apoptotic MT1-MMP$^{-/-}$ keratinocytes (24.8% ± 3.2%) compared with normal murine keratinocytes (11.2% ± 1.8%) at day 9 (Fig 2*J–L*). Our observations are consistent with the results of Nagavarapu *et al* (2002) who found that transfection of epidermal keratinocytes with an anti-sense MT1-MMP oligonucleotide correlated to reduced migration and increased apoptosis.

Several alternative mechanisms alone or together could explain the impaired early migration and increased apoptosis in MT1-MMP$^{-/-}$ keratinocytes during the late phase of epidermalization. MT1-MMP is known to cleave laminin-5, a component of the basement membrane substratum in our *ex vivo* model, directly and indirectly through pro-MMP-2 activation. In migrating mammary epithelial cells, MT1-MMP was redistributed to lamellipodia and basal surface, supposedly to enable the cells to remodel ECM molecules such as laminin-5 (Gilles *et al*, 2001). Thus, abolished generation of pro-migratory ECM molecules by the MT1-MMP-deficient keratinocytes may have attenuated migration. MT1-MMP not only hydrolyzes ECM components but also interacts with and activates integrin receptors. By processing integrin subunits, MT1-MMP facilitates integrin-dependent cell adhesion and activates the focal adhesion kinase (FAK) signaling pathway (Baciu *et al*, 2003). Migration of human keratinocytes was also attributed to induction and phosphorylation of FAK (Yurko *et al*, 2001). Furthermore, FAK activation was associated with resistance to apoptosis caused by disrupted interaction between epithelial cells and ECM (Frisch *et al*, 1996). Consequently, reduced FAK activation in MT1-MMP$^{-/-}$ keratinocytes could explain the reduced migration and increased apoptosis.

In conclusion, despite uncompromised closure of small wounds in neonatal mice with disrupted MT1-MMP gene, their skin showed impaired epidermalization on basement membrane substratum *ex vivo*. Whether our findings can be extrapolated to wound healing *in vivo* underlies further research. Nonetheless, we postulate that MT1-MMP is associated with the survival of epidermal keratinocytes.

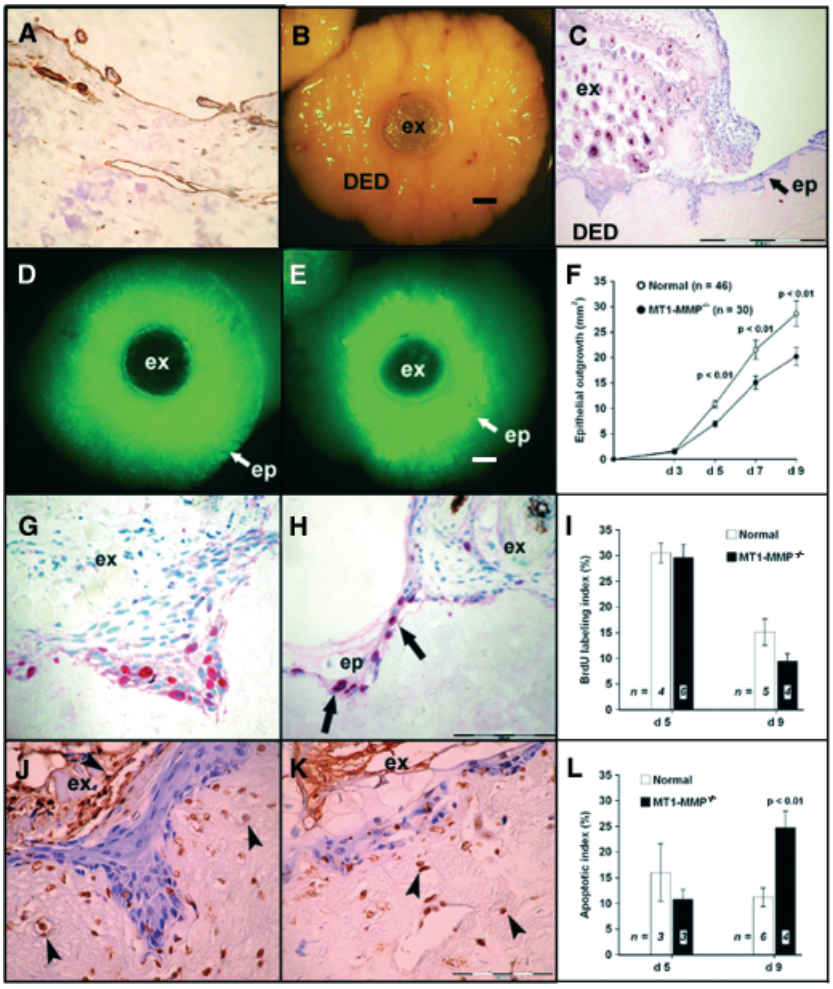

Figure 2
***Ex vivo* murine epidermalization, proliferation, and apoptosis.** (*A*) Laminin-1 immunostaining of non-cultured deepidermized dermal (DED) substrate. (*B*) Stereo-microscopical view and (*C*) skin culture composite with arrowed regenerating epithelium from a skin explant over DED substrate. (*D, E*) Green fluorescent neoepithelium with arrowed front. (*F*) Quantification of fluorescent neoepithelium from membrane-type-1 matrix metalloproteinase-deficient (MT1-MMP$^{-/-}$) skin explants from seven animals from five different litters and normal skin explants from 13 animals of the same five litters cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (3:1) containing 5 μg insulin, 0.5 μg hydrocortisone, and 10 ng epidermal growth factor per mL, 10^{-10} M cholera enterotoxin, 1.8×10^{-4} M adenine, non-essential amino acids, 10% fetal calf serum, and 100 μg streptomycin, and 100 U penicillin per mL. (*G, H*) DNA-synthesizing keratinocytes in neoepithelium (arrows) were immunodetected after a 4-h pulse with 50 μM 5-bromo-2′-deoxyuridine (BrdU) by their red nuclei (Mirastschijski *et al*, 2002). (*I*) BrdU labeling index, i.e., number of BrdU-positive cells per total number of cells × 100% in neoepithelium, determinations for the two mouse groups. (*J, K*) Apoptotic cells (brown nuclei) were immunodetected after terminal deoxytransferase-mediated digoxigenin-dUTP nick end labeling (ApopTag, Intergen Company, Purchase, New York) treatment of 5-μm paraffin tissue sections. All dermal cells (arrowheads) in DED and explant were apoptotic. (*L*) Apoptotic index, i.e., number of apoptotic cells per total number of cells × 100%, determined in neoepithelium from MT1-MMP$^{-/-}$ and MT1-MMP expressing skin explants. (*D, G, J*) Normal. (*B, C, E, H, K*) MT1-MMP$^{-/-}$. (*C, D, E, G, H, J, K*) day 9. (*F, I, L*) Data analyzed by the *t* test. (*A, C, G, H, J, K*) Same magnification. *Scale bars:* (*B, E*): 1 mm; (*C, H, K*) 100 μm; *ex*, explant; *ep*, neoepithelium; *DED*, deepidermized dermis. *Error bars:* SEM.

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